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## A Novel Cartilage Protein (CILP) Present in the Mid-zone of Human Articular Cartilage Increases with Age\*

(Received for publication, December 10, 1997, and in revised form, May 22, 1998)

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A novel, somewhat basic noncollagenous protein was purified from guanidine hydrochloride extracts of human articular cartilage using cesium chloride density gradient centrifugation, followed by ion-exchange chromatography at pH 5, and gel filtration on two serially coupled columns of Superose 6 and Superdex 200.

The protein of 91.5 kDa contains a single polypeptide chain substituted with *N*-linked oligosaccharides. It appeared unique to cartilage as studied by enzyme-linked immunosorbent assay and immunoblots of various tissue extracts. Its concentration in articular cartilages showed some variability with age being lower in young individuals. It represents a chondrocyte product, since it is synthesized by articular chondrocytes in explant cultures. Interestingly, the distribution of the protein in the articular cartilage provides important information on the nature of chondrocytes at different compartments in the tissue. Thus, chondrocytes in the middle/deeper layers of the tissue in particular, appeared to have produced the protein and deposited it in the interterritorial matrix. The protein was neither seen in the superficial nor in the deepest regions of the articular cartilage. Based on its immunolocalization we have named this protein CILP (cartilage intermediate layer protein).

Articular cartilage is a heterogeneous tissue where the cells are arranged in layers of matrix that have a different composition and function depending on their location from the articular surface to the subchondral bone (1, 2). The extracellular matrix is also arranged into compartments around the cells: pericellular (closest to the cell), territorial (extending around individual or groups of chondrocytes), and the interterritorial matrix (furthest away from the cells). The matrix is produced by the chondrocytes and contains, as major constituents, fibril-forming collagens and large aggregating proteoglycans that are assembled into highly organized structures (1, 3-5).

Collagen confers tensile properties to the tissue, whereas proteoglycans have a key role in the normal resilience and load dissipation of the cartilage. There is also a minor population of non-collagenous proteins for which no functional role has yet been identified. They may have roles in maintaining the tissue

homeostasis by the regulation of matrix assembly, cell recognition, and cell attachment. They may also have a part to play in balancing the processes of cartilage repair and degradation, as well as in disease processes where degradation outbalances repair and loss of tissue ensues.

To identify matrix constituents involved in these processes, it is imperative to identify, isolate, and characterize these matrix proteins. The present paper describes the isolation and partial characterization of a 91.5-kDa single chain protein from human articular cartilage. This protein was selected, since it appeared to change in osteoarthritis. The protein was found to be restricted in its tissue distribution to cartilage and furthermore, to specific zones within the tissue. We suggest the name cartilage intermediate layer protein (CILP)<sup>1</sup> in view of its restricted tissue distribution.

### MATERIALS AND METHODS

#### Extraction of Cartilage

Human osteoarthritic articular cartilage was obtained at surgery after total hip replacement. The cartilage was dissected clean, sliced into fine pieces, and disrupted using a high speed homogenizer (Polytron, Kinematica GmbH, Kriens-Luzern, Switzerland) in 12 volumes (w/v) of 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4, also containing a protease inhibitor mixture (5 mM benzamide hydrochloride, 0.1 M 6-aminohexanoic acid) and 5 mM *N*-ethylmaleimide. The mixture was pre-extracted for 4 h at 4 °C then centrifuged at 20,000 × *g* and 4 °C for 30 min. The pellet was extracted with 12 volumes (w/v) of 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8, containing the protease inhibitor mixture, 5 mM *N*-ethylmaleimide, and 10 mM EDTA, for 24 h at 4 °C followed by centrifugation at 20,000 × *g* at 4 °C for 30 min.

Proteins in the extract were separated from proteoglycans by CsCl density gradient centrifugation with a starting density of 1.5 g/ml under dissociative conditions in 4 M GdnHCl as described elsewhere (6). The gradient tube was divided into 4 equal fractions using a Beckman tube slicer, and the top two fractions (D3 and D4) were used for subsequent purification.

#### Protein Purification

The pooled fractions from the CsCl gradient (D3 and D4) were concentrated by ultrafiltration (PM-10 membrane, Amicon, Inc., Beverly, MA), followed by diaflow against 7 M urea, 20 mM Tris-HCl, pH 8, and then chromatographed on a column of DEAE-cellulose (5 × 10 cm, DE52, Whatman, Maidstone Chemicals, Kent, United Kingdom) equilibrated with the urea buffer. After sample loading, the column was washed with 5 bed volumes of the equilibration buffer, and eluted with a linear gradient (2 × 800 ml) from 0 to 1 M NaCl. Selected fractions were analyzed by SDS-PAGE for the presence of CILP. One pool of the protein was bound to the DEAE column and eluted at 0.04 M NaCl, while the rest of the protein was in the flow-through. This flow-through material was mixed with an equal volume of 20 mM sodium acetate, adjusted to pH 5 and the protein was further purified on a CM-52 column (1.6 × 15 cm, carboxymethyl-cellulose, Whatman Chemicals,

\* This work was supported by grants from the Swedish Medical Research Council, Konung Gustaf V's 80-årsfond, Greta och Johan Kock's stiftelser, Axel och Margaret Ax:son Johnsons stiftelse, Alfred Osterlund's stiftelse, and the Medical Faculty, Lund University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: CILP, cartilage intermediate layer protein; GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; CM, carboxymethyl; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography.

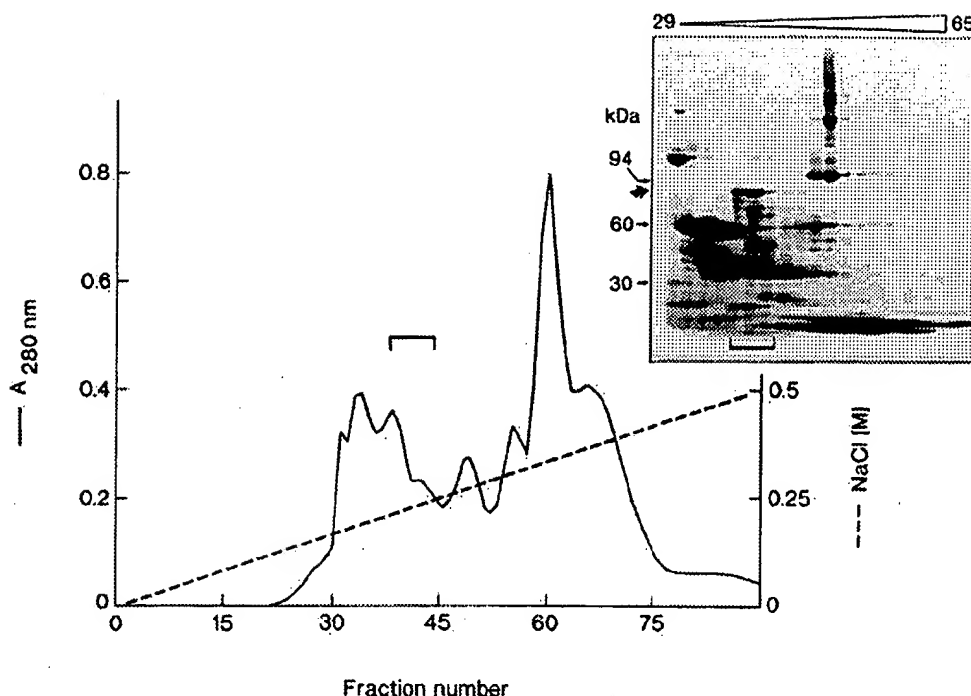


Fig. 1. CM-cellulose chromatography, pH 5. The unbound fraction from the DE52 cellulose chromatography, in 7 M urea, 20 mM Tris, pH 8, was diluted with an equal volume of 7 M urea, 20 mM sodium acetate, adjusted to pH 5, and chromatographed on a CM-cellulose column equilibrated in 7 M urea, 10 mM sodium acetate, pH 5. After washing with equilibration buffer the column was eluted with a NaCl gradient (dashed line) from 0 to 0.5 M in the urea solution. Absorbance was monitored at 280 nm (solid line). Aliquots of every two fractions, from 29 to 65, were precipitated with ethanol and electrophoresed on a 4 to 16% SDS-polyacrylamide gradient gel (inset) without previous reduction. The gel was stained for proteins with Coomassie Brilliant Blue R. The migration positions of the molecular mass markers are indicated on the left and that of CILP by a dashed arrow. The bar indicates the fractions (38–44) that were pooled for further purification.

Maidstone Kent, United Kingdom), equilibrated in 7 M urea, 10 mM sodium acetate, pH 5, at 20 ml/h. After loading the sample, the column was washed with 5 bed volumes of equilibration buffer followed by elution with a linear gradient of 0 to 0.5 M NaCl. Fractions of 10 ml were collected, monitored for protein content by measuring their absorbance at 280 nm, and analyzed by SDS-PAGE.

The fractions containing CILP were pooled and concentrated by ultrafiltration, followed by diaflow against 4 M GdnHCl, 50 mM sodium acetate, pH 5.8, and further chromatographed on two serially coupled FPLC gel filtration columns of Superose 6 and Superdex 200 (Pharmacia Biotech, Uppsala, Sweden) equilibrated and eluted at 0.2 ml/min with 4 M GdnHCl, 50 mM sodium acetate, pH 5.8. Fractions of 0.5 ml were collected and monitored for protein content by measuring their absorbance at 280 nm. Protein patterns were analyzed by SDS-PAGE.

#### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was done as described by Laemmli (7) on gradient polyacrylamide (4–16%) slab gels with a 4% polyacrylamide stacking gel. Samples of the fractions were precipitated with 10 volumes of ethanol containing 50 mM sodium acetate and recovered after centrifugation as described elsewhere (8). Precipitates were dissolved in electrophoresis sample buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 0.002% bromophenol blue, and 20% glycerol) without or with 10% 2-mercaptoethanol, incubated at 37 °C for 30 min, boiled at 100 °C for 4 min, and electrophoresed. Gels were stained with Coomassie Brilliant Blue R (Serva, Heidelberg, Germany).

The molecular mass of the purified protein was determined by running linear SDS-PAGE with concentrations ranging from 6 to 12% polyacrylamide. A Ferguson plot was constructed (9) from the relative mobilities of CILP and reference proteins. Molecular mass was determined from the retardation coefficients ( $K_r$ ) as described by Banker and Cotman (10).

#### Immunoassays

**Preparation of Antibodies**—Antibodies were raised in rabbits by using CILP purified from the gel filtration separation. The protein (100 µg) was solubilized in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4, mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected at multiple sites subcutaneously

in the back. Booster doses (100 µg) in Freund's incomplete adjuvant (Difco) were given twice, at 1-month intervals.

**Immunoblot**—Articular cartilage from different mammalian species: bovine, equine, canine, murine (Swarm rat chondrosarcoma), and human, were extracted with 15 volumes of 4 M GdnHCl containing protease inhibitors, as described above. Aliquots of the extracts corresponding to 2 mg of tissue wet weight, were precipitated twice with 10 volumes of ethanol as described (8). Extracts were electrophoresed on two identical 4–16% gradient SDS-PAGE under nonreducing conditions, with a sample of purified CILP electrophoresed for comparison. One gel was stained for protein with Coomassie Brilliant Blue-R. Proteins were transferred from the other gel to a nitrocellulose membrane (Hybond-C, Amersham, United Kingdom) by diffusion (11) for immunodetection. The membrane was blocked for 1 h with 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.2% Tween 20, and incubated with the antibodies to CILP diluted 1:500 in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.2% Tween 20 containing also 0.1% BSA. Bound antibodies were detected with anti-rabbit IgG peroxidase conjugated (Dakopatts, Copenhagen, Denmark) diluted 1:500 in the same buffer as the primary antibody. Enzyme activity was detected using  $H_2O_2$ /diaminobenzidine containing cobalt chloride and ammonium nickel sulfate (12).

#### Enzyme-linked Immunosorbent Assay (ELISA)

The assays were essentially done as described previously (13). CILP was coated onto NUNC  $\gamma$ -irradiated polystyrene microtiter plates (Immunoplate I, NUNC, Roskilde, Denmark) at 0.5 µg/ml in 4 M GdnHCl, 50 mM sodium carbonate, pH 10, by incubation overnight at room temperature. After rinsing with 0.15 M sodium chloride, 0.05% (w/v) Tween 20, the plates were aftercoated with 2 mg/ml bovine serum albumin (Serva, Fine Biochemicals, Heidelberg, Germany) in phosphate-buffered saline, pH 7.4, for 1 h.

Samples of human tissues were extracted with 15 volumes of 4 M GdnHCl containing protease inhibitors as described above. The extracts were precipitated twice with ethanol (8) and resuspended in 0.8% SDS in 10 mM phosphate-buffered saline, pH 7.4, to give stock solutions corresponding to 4 mg of the original tissue wet weight per ml. Dilutions were made from these stock solutions and added to an equal volume of antiserum in 4% (v/v) Triton X-100, 0.01 M sodium phosphate, pH 7.4. After preincubation for 1 h at room temperature this mixture was added

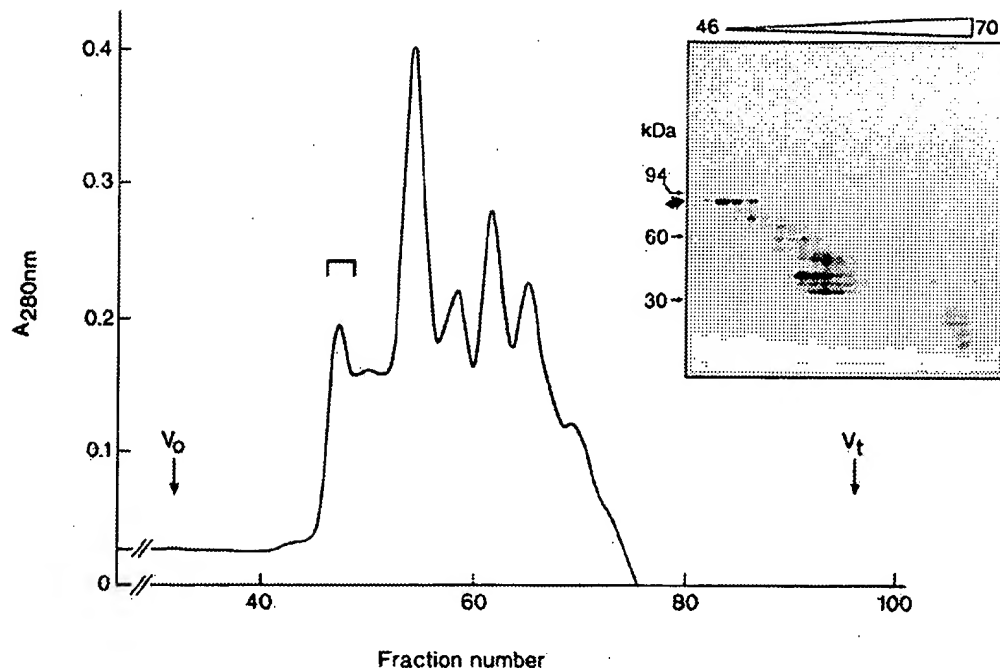


Fig. 2. Gel filtration chromatography. The pooled fractions from the CM-cellulose column were concentrated by ultrafiltration, followed by diaflow against 4 M GuHCl, 50 mM sodium acetate, pH 5.8, and chromatographed on two tandemly arranged FPLC columns of Superose 6 and Superdex 200. Absorbance was monitored at 280 nm. The peak containing CILP is indicated by a bar (fractions 47–50). Aliquots of each fraction (46–70) were precipitated with ethanol and electrophoresed on a 4 to 16% SDS-polyacrylamide gradient gel without previous reduction (inset). The gel was stained for proteins with Coomassie Brilliant Blue R. The migration positions of the molecular mass markers are indicated on the left and that of CILP by a curved arrow (47–50).

to the coated wells of the microtiter plates. After 1 h plates were rinsed and bound antibodies were detected using a swine anti-rabbit alkaline phosphatase conjugate (Orion Diagnostica, Helsinki, Finland) with *p*-nitrophenyl phosphate (Sigma) as the substrate.

A standard curve using purified CILP in 0.8% (w/v) SDS, 10 mM phosphate buffered saline, pH 7.4, was included in each microtiter plate. All samples were analyzed in triplicate and the mean value was used for calculations.

#### Immunohistochemistry

Normal appearing human articular cartilage from a 38-year-old hip joint was obtained from autopsy. Full-depth plugs of cartilage, 4 mm in diameter, were excised using a cork borer. The cartilage was frozen, embedded in O.C.T. compound (Tissue Teck II, Miles Laboratories, Naperville, IL) and sectioned at  $-25^{\circ}\text{C}$  in a cryostat. Cryosections (5  $\mu\text{m}$ ) were transferred onto gelatin-coated glass slides, dried at room temperature for 2 h, fixed at  $4^{\circ}\text{C}$  with cold acetone for 10 min, and rehydrated in phosphate-buffered saline (PBS). To increase antibody permeability, the sections were digested for 30 min at room temperature with 2 mg/ml hyaluronidase (from bovine testes, type I, Sigma) in PBS, pH 5. Endogenous peroxidase was quenched by incubating in PBS containing 1% (v/v)  $\text{H}_2\text{O}_2$  for 20 min at room temperature. After rinsing three times with PBS, 0.1% (w/v) BSA, each section was incubated with goat serum diluted 1:70 in PBS, 0.1% (w/v) BSA for 20 min to reduce nonspecific binding. Sections were then incubated with the primary antibody against CILP (diluted 1:800 with PBS, 0.1% BSA) or with preimmune rabbit serum (diluted 1:400 with PBS, 0.01% BSA) at  $4^{\circ}\text{C}$  overnight in a moist chamber. After rinsing three times with PBS, 0.01% BSA, the sections were treated with biotinylated second antibody (diluted 1:200) and avidin peroxidase conjugate using the vectastain ABC kit (Vector Laboratories, Burlingame, CA), according to the protocol of the manufacturer.

#### CILP Content in Different Layers of Articular Cartilage

Normal appearing human articular cartilage was obtained from a 38-year femoral head at post-mortem. Full-depth plugs of cartilage, 4 mm in diameter, were excised using a cork borer and sectioned in a cryostat at  $-25^{\circ}\text{C}$ . Slices 20  $\mu\text{m}$  thick were cut parallel to the cartilage surface, and each 10 consecutive sections were pooled to represent 200  $\mu\text{m}$ . This procedure provided 9 pools from the articular surface to the subchondral bone. The slices were extracted with 200  $\mu\text{l}$  of 4 M GdnHCl,

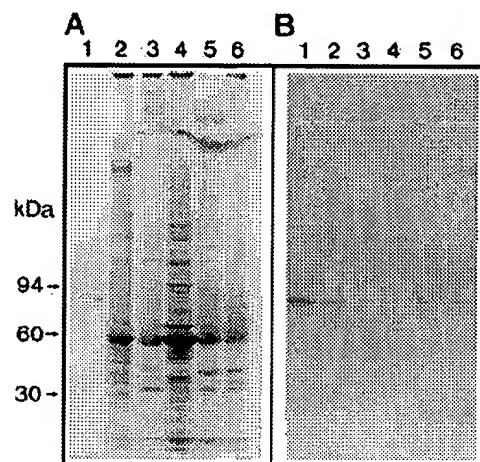
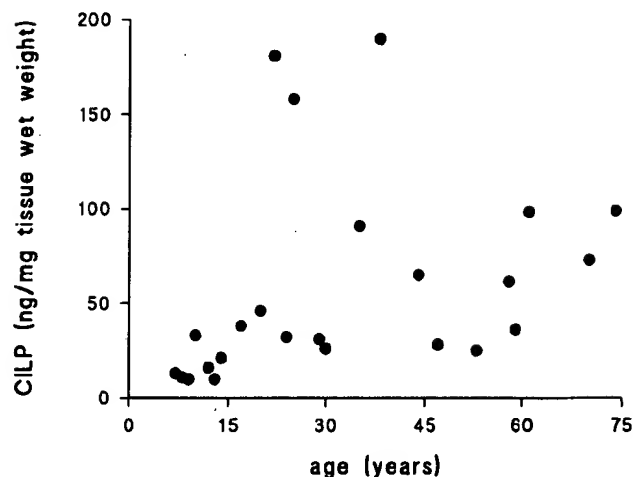
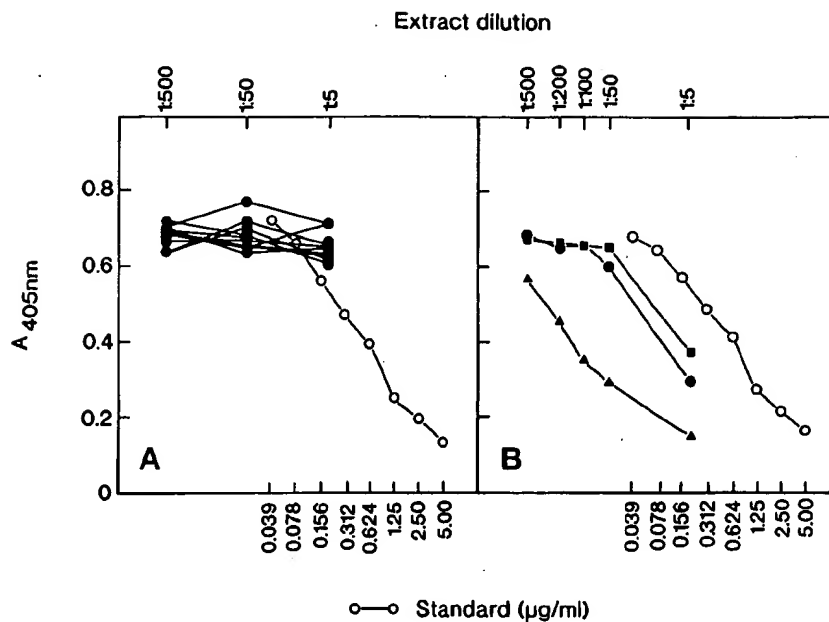


Fig. 3. Immunoblot analysis of CILP in extracts of articular cartilage of different species. Samples of articular cartilage from various species and Swarm rat chondrosarcoma were extracted with 15 volumes of 4 M GdnHCl, containing a number of proteinase inhibitors including *N*-ethylmaleimide. Aliquots of the extracts, corresponding to 2 mg of tissue wet weight, were precipitated with ethanol and electrophoresed on two identical 4–16% SDS-polyacrylamide gradient gels without prior reduction. A purified fraction of CILP was included as a standard. One gel was stained for proteins with Coomassie Brilliant Blue R (A). Proteins were transferred from the gel to nitrocellulose filters for immunodetection (B). The membrane was probed with the antiserum against the human CILP. Bound antibody was detected with a second peroxidase-conjugated swine IgG directed against rabbit IgG and developed with  $\text{H}_2\text{O}_2$ /diaminobenzidine (B). The migration positions of the molecular mass markers are indicated on the left. Lanes: 1, purified CILP; 2, human; 3, cow; 4, rat chondrosarcoma; 5, dog; 6, horse.

0.05 M sodium acetate, pH 5.8, containing the protease inhibitor mixture and 5 mM *N*-ethylmaleimide as described. The extracts were precipitated twice with ethanol (8) and the CILP content in each pool was determined by ELISA as described.

**FIG. 4. Enzyme-linked immunosorbent assay of CILP in human tissues.** Tissues were extracted with 15 volumes of 4 M GdnHCl, containing proteinase inhibitors including *N*-ethylmaleimide. Aliquots of the extracts corresponding to 4 mg of tissue wet weight, were precipitated with ethanol, resuspended in 10 mM phosphate-buffered saline, pH 7.4, containing 0.8% SDS and diluted as indicated. ELISA was performed as described under "Materials and Methods." **A**, noncartilaginous tissues: liver, kidney, intestine, lung, skin, aorta, muscle, tendon, bone. **B**, cartilages: tracheal (■), articular (●), and costal (▲). Purified CILP was used as standard (○) at the indicated concentration in each set of assays.

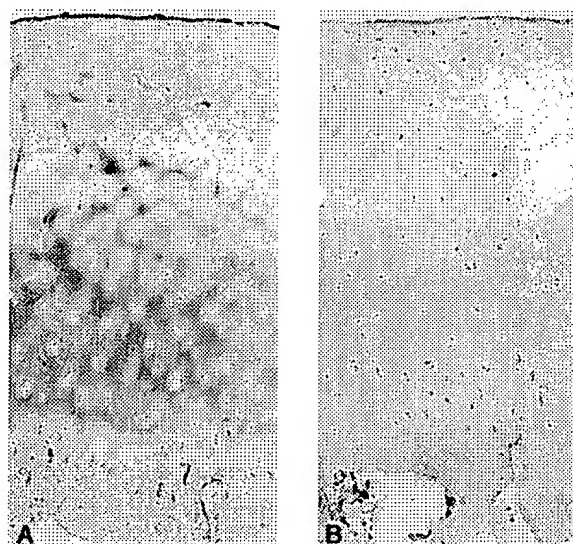


**FIG. 5. CILP content in articular cartilages from individuals of different ages.** Cartilages were processed as described in the legend to Fig. 4. A standard curve using a purified CILP was included in each microtiter plate. All the samples were analyzed in triplicate, and the mean value was used for calculations.

#### Immunoprecipitation

Human articular cartilage (femoral head) was obtained at surgery for hip replacement, dissected under sterile conditions, and placed (25 mg of tissue/ml medium) in Ham's F-12 culture medium (Life Technologies, Inc., Grand Island, NY), pH 7.4, supplemented with 10% (v/v) fetal calf serum and 25  $\mu$ g/ml ascorbate. The explants were metabolically labeled with 50  $\mu$ Ci/ml [ $^3$ H]leucine and 25  $\mu$ Ci/ml [ $^{35}$ S]sulfate (Amersham International, Bucks, UK) for 4 h at 37 °C under 5%  $\text{CO}_2$ , 95% air. After labeling the explants were washed with medium without isotopes, wiped dry and extracted with 4 M GdnHCl, as described above. An aliquot of the extract was precipitated with ethanol and dissolved in 60  $\mu$ l of 10 mM phosphate-buffered saline, pH 7.4, containing 0.8% (w/v) SDS. To bind excess SDS the sample was then mixed with 60  $\mu$ l of 2% (v/v) Triton X-100 in the same buffer. Rabbit immune serum (30  $\mu$ l) was immediately added and the sample was incubated overnight at 4 °C. Immunoglobulins and bound antigen were then adsorbed onto protein-A Sepharose (30  $\mu$ l) (Pharmacia, Uppsala, Sweden) by incubation for 4 h at 4 °C. Bound material was recovered by centrifugation, washed, resuspended in electrophoresis buffer, and electrophoresed as above.

Radiolabeled proteins were detected by fluorography. The polyacrylamide gels were washed in distilled water for 30 min, soaked in 5 volumes of 1.3 M sodium salicylate for 35 min, dried on a gel drier (LKB



**FIG. 6. Immunolocalization of CILP in human articular cartilage (hip).** Cryosections were immunoreacted with polyclonal antibodies to CILP, followed by peroxidase-conjugated anti-rabbit antibodies. The sections had been predigested with testicular hyaluronidase before incubation with antibodies against CILP (A) or with the preimmune antiserum (B). Magnification was  $\times 40$ .

Bromma, Sweden) and, exposed to a preflashed Kodak XAR-5 film for 3–4 weeks at  $-80$  °C.

#### *N*-Glycosidase F and *O*-Glycosidase Digestions

Samples to be digested were precipitated with ethanol. For the *N*-glycosidase F digestion the samples were resuspended in 0.1 M Tris-HCl, pH 6.8, containing 0.1% SDS, and incubated in a boiling water bath for 3 min. Then an equal volume of 0.125 M Tris-HCl, pH 6.8, was added, plus 5  $\mu$ l of 0.5% Nonidet P-40, 1  $\mu$ g of trypsin inhibitor (from chicken egg white type II-0, Sigma), and 1 unit of enzyme (PNGase F, Boehringer Mannheim, GMBL, Germany). Samples to be digested with *O*-glycosidase were resuspended in 15 mM sodium cacodylate, pH 6.0. Trypsin inhibitor (1  $\mu$ g) was added to the reaction mixture and 0.5 milliunits of enzyme (Boehringer Mannheim). Deglycosylation was overnight in a water bath at 37 °C.

An aliquot of the mixtures before and after digestion was diluted with sample buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 0.002% bromphenol blue, and 20% glycerol), boiled at 100 °C for 4 min, and electro-

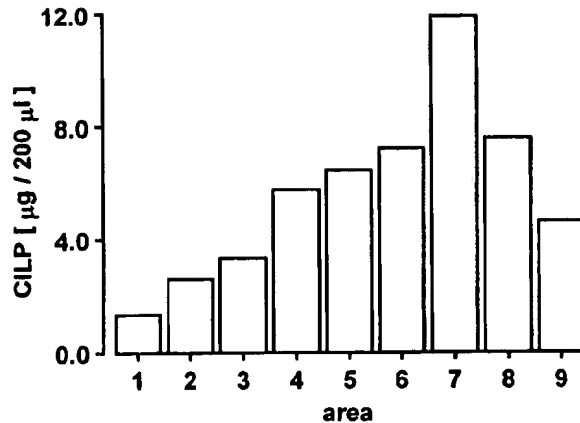


FIG. 7. CILP content in different layers of human articular cartilage. Samples of full-depth cartilage were sectioned parallel to the cartilage surface into 20- $\mu$ m thick slices and each 10 consecutive sections were pooled to represent 200  $\mu$ m. Slices were extracted with 4 M GdnHCl for 24 h at 4 °C. Extracts were precipitated twice with ethanol, resuspended in 10 mM phosphate-buffered saline, pH 7.4, containing 0.8% SDS and analyzed by ELISA.

phoresed on a linear 8% acrylamide gel. Proteins were visualized by staining with Coomassie Brilliant Blue R (Serva, Heidelberg, Germany).

#### RESULTS

**Isolation of CILP**—A 91.5-kDa monomeric protein was isolated from GdnHCl extracts of human articular cartilage. The initial step included a dissociative CsCl gradient centrifugation in order to separate the matrix proteins from the bulk of proteoglycans. The lower density fractions of the gradient (D3 plus D4) were combined and used as the source of CILP. This fraction was chromatographed on DEAE cellulose at pH 6, where a pool of CILP appeared in the flow-through. This pool was applied on a CM-52 column equilibrated at pH 5 and eluted with a gradient of 0–0.5 M NaCl. Analysis of selected fractions by SDS-PAGE under nonreducing conditions showed that CILP eluted at 0.22 M NaCl in a small peak together with a large number of lower molecular weight components (Fig. 1). The fractions (38–44) containing CILP were combined and brought into 4 M GdnHCl, 50 mM sodium acetate, pH 5.8, by diaflow and was then further purified on serially coupled FPLC gel filtration columns of Superose 6/Superdex 200 FPLC. CILP eluted in a small peak over four fractions (47–50, Fig. 2), two of which were free of other proteins as judged by the SDS-PAGE (Fig. 2, inset). By this procedure a yield of 129  $\mu$ g of CILP per gram of tissue wet weight was obtained. This yield from osteoarthritic cartilage, however, represents an underestimation of the total, since the losses in the procedure including the CILP bound to the DEAE column are not accounted for.

The relative mobility of CILP on SDS-PAGE was not significantly affected by reduction, which suggested a monomeric protein (data not shown). The molecular mass of CILP was estimated after electrophoresis of the protein on 6, 8, 10, and 12% SDS-PAGE (6, 7). The Cotman plot suggested an ideal behavior and the molecular mass determined was 91,500 Da (data not shown).

Early attempts to purify CILP from human articular cartilage involved direct extraction of the cartilage with the GdnHCl solution. However, the abundant albumin in the preparation masked a pool of CILP that was bound to the DEAE column. This pool eluted at a very low salt concentration (0.04 M NaCl), while the remainder of CILP appeared in the flow-through as discussed above. Preliminary studies were done using protein in this pool purified by CM-52 chromatography and gel filtration. In a subsequent study investigating the component which

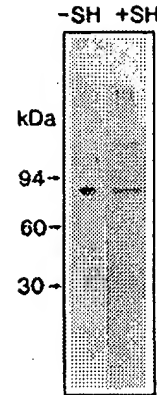


FIG. 8. Synthesis of CILP by articular chondrocytes in explant culture. Human articular cartilage explants were incubated with medium containing [ $^3$ H]leucine and [ $^{35}$ S]sulfate. After extraction with 4 M GdnHCl, the extract was precipitated with ethanol, resuspended in Tris-buffered saline containing 0.2% (v/v) Tween, and immunoprecipitated with polyclonal antibodies specific for CILP. The immunoprecipitated material was electrophoresed on a 4–16% SDS-polyacrylamide gradient gel without (-SH) and with reduction (+SH). Radiolabeled components were visualized by fluorography.

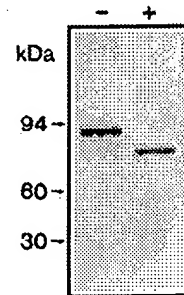


FIG. 9. Deglycosylation of CILP. A sample of CILP was subjected to *N*-glycosidase F digestion. Products of reaction were resolved by 8% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250.

bound to DEAE, the tissue was pre-extracted with phosphate-buffered saline at pH 7.4, containing the protease inhibitor mixture, to remove the albumin and other proteins not tightly held in the cartilage matrix.

The two pools of CILP, *i.e.* the one bound to the DEAE and the one in the flow-through, showed identical peptide patterns after trypsin digestion when analyzed by reverse phase high performance liquid chromatography (C18) (data not shown). The two protein variants also appeared to have a similar content of *N*-linked oligosaccharides since their respective mobilities upon SDS-PAGE before and after *N*-glycanase digestion were identical (data not shown). No further experiments were done to elucidate the difference between the two proteins. All the biochemical studies were done using the protein pool from the CM-cellulose chromatography (Fig. 2).

**Specificity of Antibodies against CILP**—Antibodies against CILP were raised in rabbits by using the purest fractions from the gel filtration chromatography. To determine the specificity of the antibodies, an aliquot of the guanidine hydrochloride extracts from different species (human, bovine, murine, canine, and equine) were precipitated with ethanol, electrophoresed on SDS-PAGE, and transferred onto nitrocellulose membranes. An immunoreactive band corresponding to the relative mobility of CILP was detected in the extracts (Fig. 3). No positive staining was, however, observed in the extract of the Swarm rat chondrosarcoma. There were differences in the relative intensities of the band in the various tissues, although the

protein staining indicate similar load of the extracts onto the different lanes. This may result from limited cross-reactivity or indicate a variable abundance of the protein.

**Distribution of CILP Among Human Tissues and with Age Variation**—GdnHCl extracts were prepared from various tissues, and the presence of CILP was determined by using an enzyme-linked immunosorbent assay developed (Fig. 4). All the cartilage extracts analyzed gave inhibition curves parallel to the standard showing that the assay actually measured the same protein with no interfering substances. The concentration of CILP was calculated to be 129 ng/mg tissue wet weight for normal articular cartilage (from a finger joint), 62 ng/mg for tracheal cartilage, and 983 ng/mg for rib cartilage. It was apparent that the non-cartilaginous tissues analyzed (liver, kidney, intestine, lung, skin, aorta, muscle, tendon, and bone) did not contain detectable amounts of the protein.

Analysis of the CILP content in articular cartilages from individuals of different ages showed considerably lower values in the younger individuals (Fig. 5). After cessation of growth, the levels of the protein were quite variable, but in general they were higher than in the younger individuals.

**Immunolocalization**—The distribution of CILP in human articular cartilage was studied by immunohistochemistry applying the avidin-biotin-peroxidase method using cryosections. The protein was detected primarily in the middle zone, while it was less prominent or absent at the superficial and the very deepest zones of the articular cartilage (Fig. 6). The antibodies primarily stained the interterritorial matrix of the tissue, while low or no reactivity was seen in the pericellular matrix of the chondrocytes. Based on its immunolocalization we have named this protein CILP (cartilage intermediate layer protein).

**CILP Content in Different Layers of Articular Cartilage**—To further characterize the CILP localization within the articular cartilage, sections parallel to the cartilage surface were made. Nine layers of 250  $\mu$ m from the articular surface to the subchondral bone were obtained, and the CILP content was determined by ELISA as shown in Fig. 7. Even though there were detectable amounts of CILP through the cartilage depth, the content was higher in the middle layers.

**Immunoprecipitation**—To determine whether the protein is a true cartilage constituent synthesized by the chondrocytes, human hip articular cartilage explants were metabolically labeled with [ $^3$ H]leucine and [ $^{35}$ S]sulfate. After extraction the protein was immunoprecipitated. As it is shown in Fig. 8, a major band with a relative mobility corresponding to CILP was identified by fluorography after SDS-PAGE under reduced and nonreduced conditions. However, after reduction two higher molecular weight components were also immunoprecipitated. They may represent either aggregates or possibly cross-reactivity with some related proteins or nonspecific reactivity that is sometimes observed at this position with other rabbit antisera (data not shown).

**N-Glycosidase F and O-Glycosidase Digestions**—CILP contains N-linked oligosaccharides as shown by a significant change in its relative mobility after N-glycosidase F digestion (Fig. 9). The change in mobility gives an estimate of the oligosaccharide content to represent as much as 10% of the molecular mass of the protein. No apparent change in its mobility was observed after O-glycosidase digestion (data not shown).

#### DISCUSSION

The functional demands on different parts of articular cartilage are not fully understood. It is, however, known that different parts of the tissue have a different composition and structure. Thus, in the superficial region of the tissue, the collagen fibers are thinner and the relative abundance of aggrecan is much lower. In contrast, contents of decorin and

COMP are higher than in the remainder of the tissue. Cells in the deeper layer elaborate thicker collagen fibers that are arranged in a different direction to those in the surface layers and the deep zone also has a higher aggrecan content. However, there have been no previous descriptions of a protein, like CILP, that can be used to define an intermediate layer of the tissue. It is an enigma why a specific protein is required to satisfy the functional demands of the tissue in this layer, and the characteristics of the protein offers no explanation. It is clear that CILP is synthesized and deposited in the extracellular matrix by the chondrocytes. Its absence from other tissues is also of interest and is further evidence suggesting that the protein has a specific function in cartilage. The restricted distribution of the protein to the interterritorial compartment also indicates that it has a possible role in maintaining the structure of the tissue rather than in the regulation of cellular activities. It is of interest to note that CILP is one of four proteins whose expression is enhanced in the early stages of human osteoarthritis.<sup>2</sup> This may indicate a specific role for the protein in tissue repair. The high concentration of the protein in rib cartilage is intriguing. Why this particular cartilage is highly enriched in CILP will be the objective of future studies. Similarly, the relatively low levels in tracheal cartilage should also provide clues to the function of CILP. These preliminary findings may be taken to indicate that compressive load is a factor controlling the tissue content of the protein. Future work will investigate the role of the protein in tissue biology.

CILP has an apparent molecular mass similar to that of, e.g. 92-kDa gelatinase. In contrast to CILP this proteinase is, however, not restricted to cartilage and to the interterritorial matrix. Further support for CILP as a novel protein was obtained by cDNA cloning and deduced amino acid sequence (14). CILP contains N-glycosidically linked oligosaccharides, whereas there was no indication of O-glycosidically linked sugars. An estimated alteration in mass of 10% of the total upon removal of these oligosaccharides, indicates that 3–5 such structures are present.

CILP levels in articular cartilage increases with age. Similarly, there is considerable evidence to support the accumulation of matrix aggrecan during normal aging of articular cartilage. The increasing concentration of CILP with age may, like in the case of aggrecan, reflect a slow turnover of the protein, particularly in the interterritorial matrix.

**Acknowledgments**—We thank Ros-Mari Sandfalk and Lelis Zunino for technical assistance.

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<sup>2</sup> P. Lorenzo, M. T. Bayliss, and D. Heinegård, unpublished data.